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Battaglia, A ; Pak, K ; Brors, D ; Bodmer, D ; Frangos, J A ; Ryan, A F

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## INVOLVEMENT OF RAS ACTIVATION IN TOXIC HAIR CELL DAMAGE OF THE MAMMALIAN COCHLEA

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**Abstract**—To identify possible intracellular mediators of hair cell (HC) death due to ototoxins, we treated basal-turn, neonatal, rat HCs *in vitro* with several intracellular signaling inhibitors, prior to and during gentamicin exposure. The general guanine nucleotide-binding protein (G-protein) inhibitor, GDP- $\beta$ S (1 mM), provided potent HC protection, suggesting involvement of G-proteins in the intracellular pathway linking gentamicin exposure to HC death. ADP- $\beta$ S had minimal effect, indicating that the protection is specific to guanosine diphosphate (GDP)-binding, rather than a general reaction to nucleotides. Azido-GTP<sup>32</sup> photolabeling and gel electrophoresis indicated activation of an approximately 21 kDa G-protein in HCs after exposure to gentamicin. Spectroscopic analysis of peptide fragments from this band matched its sequence with H-Ras. The Ras inhibitors B581 (50  $\mu$ M) and FTI-277 (10  $\mu$ M) provided potent protection against damage and reduced c-Jun activation in HC nuclei, suggesting that activation of Ras is functionally involved in damage to these cells due to gentamicin. © 2003 IBRO. Published by Elsevier Ltd. All rights reserved.

**Key words:** FTI-277, inner ear, gentamicin, G-protein, c-Jun phosphorylation, signal transduction.

Significant hearing loss occurs in about 10% of the population, and in its severe form can produce devastating isolation. In contrast to conductive hearing loss, which is produced by pathologies in the external and/or middle ear,

sensorineural hearing loss (SNHL) is associated with damage to cochlear hair cells (HCs) or neurons. Because HCs are the most vulnerable elements in the cochlea, damage to them underlies most forms of SNHL.

SNHL can result from a variety of causes, including genetic disorders, infectious disease, overexposure to intense sound and certain drugs. The most prevalent cause of SNHL is aging. SNHL is often progressive, and mostly irreversible. During the past several years, significant progress in understanding SNHL has been made (see Ding et al., 1999; Shulman, 1999; Ryan, 2000 for reviews). Oxidative stress with the production of free radicals (Schacht, 1999; Sha et al., 2001), activation of stress pathways (Pirvola et al., 2000) and apoptosis (Huang et al., 2000), have been implicated in HC damage and death.

Recently it has been demonstrated that c-Jun-N-terminal kinases (JNK) are activated in HCs after gentamicin exposure and that an inhibitor of mixed-lineage kinase, upstream activators of JNKs, protected HCs from toxicity (Pirvola et al., 2000). The JNKs are a member of the mitogen-activated group of protein kinases (MAPKs) that are important signal transducing enzymes involved in many facets of cellular regulation including apoptosis (Chang and Karin, 2001). However, intracellular signals that lead to JNK activation in HCs have yet to be defined.

Among the elements leading to JNK activation in other cells are the guanine nucleotide-binding proteins (G-proteins). For example, activation of Ras in certain cell types has been shown to be sufficient to activate JNK (Rausch and Marshall, 1997). Members of the Rho family including Rho, Rac and Cdc42 have also been implicated in JNK activation (Bagrodia et al., 1995; Minden et al., 1995). In addition, direct activation of G-proteins by free radicals has been described in other cell systems (Perez-Sala and Rebollo, 1999) and there are substantial data linking G-proteins with apoptosis (Mayo et al., 1997). In this study we addressed the role of GTPases in gentamicin-induced toxicity signaling.

## EXPERIMENTAL PROCEDURES

### Tissue culture

Explants of sensory epithelium from the basal cochlear turn of postnatal day 5 (p5) Sprague–Dawley rats were isolated using the methods of Van De Water and Sobkowicz (Van de Water and Ruben, 1974; Sobkowicz et al., 1993). Explants were maintained in Dulbecco's Modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 30 U/ml penicillin, to which HEPES buffer had been added to a concentration of 25 mM to stabilize pH and the glucose concentration increased to 6 mg/ml to enhance neuronal survival (Gibco, BRL, Grand Island, NY, USA). Each 15 mm

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**Abbreviations:** ADP, adenosine diphosphate; CDC-42, cell division cycle protein-42; DHB, 2,5-dihydroxybenzoic acid; DMEM, Dulbecco's Modified Eagle's medium; DTT, dithiothreitol; ERK, extra cellular regulated kinase; FBS, fetal bovine serum; Ftase, farnesyl-transferase; G-proteins, guanine nucleotide-binding proteins; GDP, guanosine diphosphate; GTP, guanosine triphosphate; HC, hair cell; IHCs, inner hair cells; IMAC, immobilized metal ( $\text{Fe}^{+3}$  or  $\text{Al}^{+3}$ ) affinity chromatography; JNK, c-Jun-N-terminal kinases; MALDI, matrix-assisted laser desorption ionization; MAPKs, mitogen activated group of protein kinases; MEK, MAPK kinase; MEKK, MEK kinase; MKK, MAPK kinase; OC, organ of Corti; OHCs, outer hair cells; PBS, phosphate-buffered saline; [ $\gamma^{32}\text{P}$ ]GTP- $\gamma\text{AA}$ , guanosine 5'-[ $\gamma^{32}\text{P}$ ] triphosphate [ $\gamma$ ] 4-azidoanilide; Ras, retrovirus associated DNA sequence; Rho, Ras related homolog; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SNHL, sensorineural hearing loss; U0126, 1,4-diamino-2,3-dicyano-1,4-bis-[2-aminophenylthio]butadiene.

dish containing 250  $\mu$ l of medium was maintained in an incubator at 37 °C with 5% CO<sub>2</sub> and 95% humidity. Gentamicin alone cultures were maintained in this initial medium for 12–24 h and then exposed to medium containing 35  $\mu$ M gentamicin (Sigma, St. Louis, MO, USA) for 48 h. This concentration has been found to result in approximately 50% loss of outer HCs (OHCs), and was used to permit increases or decreases in OHC damage to be detected. For all inhibitor studies, cultures were maintained overnight (12 h) with a test substance. This was to permit the substance to penetrate the HC membrane. They were then challenged with medium containing 35  $\mu$ M gentamicin plus the test substance for an additional 48 h, followed by fixation and evaluation. The doses of test substances (Cal-Biochem, San Diego, CA, USA; Sigma) employed were based on published 50% inhibitory concentrations (IC<sub>50</sub>) in other whole-cell systems: the farnesyl-transferase (Ftase)-1-inhibitor B581 (5, 50  $\mu$ M); the more specific and cell-permeable Ftase inhibitor FTI-277 (1, 2, 5, 10, 25  $\mu$ M); GDP- $\beta$ S and ADP- $\beta$ S (1  $\mu$ M; Lerner et al., 1995; Ui et al., 1995; Burke et al., 1996; Slee et al., 1996; Leonard, 1997; Eysers et al., 1998; Humpf et al., 1998; Newton et al., 2000). GDP- $\beta$ S, a competitive inhibitor of GTP, and ADP- $\beta$ S, a structurally similar molecule that does not interact with GTP binding sites, are known to have low but similar permeabilities in intact cells after 3–4 h (Kuchan et al., 1994). U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene) is cell permeable and a very specific MEK inhibitor at micromolar concentrations. U0126 (IC<sub>50</sub> approximately 1.0  $\mu$ M) has been shown not to inhibit protein kinase C, Raf, MEKK, ERK, JNK, MKK3, 4, and 6, and Cdk2 and 4 (Alessi et al., 1995; Newton et al., 2000). U0126 was used at 1  $\mu$ M. We and others have shown this concentration to be non-toxic to many cell types (e.g. Alessi et al., 1995; Newton et al., 2000; Aletsee et al., 2001; Palacios et al., 2002). U0124 is a structurally similar but inactive compound that is often used as a control for U0126. The highest dose of each inhibitor used was used in combination with 50  $\mu$ M gentamicin on *E. coli* cultures, as a control for physical interaction with the antibiotic. None had any effect on bactericidal activity (data not shown).

### Phalloidin assays

At the end of culture, the free-floating explants were fixed with 4% paraformaldehyde and 1% glutaraldehyde in phosphate-buffered saline (PBS) for 45 min at 20 °C, then permeabilized with 1% Tween 20 (polyoxyethylene sorbitan monolaurate) in PBS at 20 °C for 30 min. The explants were then stained with a conjugated phalloidin-rhodamine probe in PBS at 20 °C for 45 min, washed twice with PBS and mounted between two coverslips in a 1:3 mixture of PBS/glycerol. Phalloidin-rhodamine stains the F-actin of the stereocilia and cuticular plate of viable HCs (Thorne et al., 1987). It also lightly outlines the body of HCs by binding to the actin that is associated with the inner surface of the cell membrane (Thorne et al., 1987). Because one of the earliest signs of morphologic injury is damage to stereocilia, it is readily detected by phalloidin staining. Subsequent apical vacuolization, swelling of the HC, damage to the cuticular plate and finally HC death can also be observed (Raphael et al., 1994). The phalloidin assay has been widely used to assess the integrity of HCs (Meiteles and Raphael, 1994; Raphael et al., 1994). Explants were imaged by light and fluorescence microscopy and by laser confocal microscopy.

### Assessment of HC survival

HCs were characterized as missing if no stereocilia or cuticular plate in the appropriate location was observed. Quantitative results were obtained by evaluating the thirty OHCs associated with 10 inner HCs (IHCs) in a given microscope field. The average of four separate counts was used to represent each culture and only those cultures for which four separate counts could be obtained

were included in the study. Six cultures were used for each condition tested.

### Phospho-c-Jun immunohistochemistry

The basal turn of the organ of Corti (OC) was dissected and cultured as described above. Gentamicin alone explants were exposed to 35  $\mu$ M gentamicin for 20 h. Experimental cultures were pretreated with FTI-277 (35  $\mu$ M) for 12 h, then with gentamicin and FTI-277 for 20 h. Normal control explants were maintained in culture medium alone. FTI-277 alone explants were maintained in culture media containing 10  $\mu$ M FTI-277 alone. At the end of the culturing period, the explants were fixed as described above, permeabilized with 5% Triton X-100 in PBS with 10% FBS for 30 min. The explants were washed twice with PBS and incubated in 1% donkey serum, then immunostained with a polyclonal rabbit anti-phospho-c-Jun antibody (Ser-73; 1:50; Cell-Signaling). The explants were washed twice with PBS, and incubated with a FITC conjugated donkey anti-rabbit IgG (1:100; Jackson ImmunoResearch, Bar Harbor, ME, USA) for 2.5 h, washed twice with PBS and stained with the conjugated phalloidin-rhodamine probe for 1 h, washed twice in PBS and visualized on a fluorescent microscope with either red filter (for the phalloidin-rhodamine staining, excitation/emission wavelength 596 nm/615 nm) or green filter (for the FITC staining, excitation/emission wavelength 490 nm/525 nm). The primary antibody was replaced with PBS as a control for non-specific binding.

### G-protein photoaffinity labeling, isolation and sequence analysis

To determine whether ototoxins activate G-proteins, photoaffinity labeling using a hydrolysis-resistant, photoaffinity GTP azido-analog, guanosine 5'-[ $\gamma$ -<sup>32</sup>P] triphosphate [ $\gamma$ ] 4-azidoanilide ([ $\gamma$ -<sup>32</sup>P]GTP- $\gamma$ AA) was employed. In photoaffinity G-protein labeling, a covalent linkage is created between the UV-sensitive, [ $\gamma$ -<sup>32</sup>P]GTP- $\gamma$ AA probe and activated G-proteins, in which the GTP analog occupies the GTP binding site. Thus, G-proteins that are activated after exposure to ototoxins in cells loaded with the [ $\gamma$ -<sup>32</sup>P]GTP- $\gamma$ AA probe can be irreversibly labeled and can subsequently be analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography (Basu and Modak, 1987). Once photolabeled, the proteins can also be purified through the use of immobilized metal (Fe<sup>+3</sup> or Al<sup>+3</sup>) affinity chromatography (IMAC). Immobilized Fe<sup>+3</sup> or Al<sup>+3</sup> selectively binds phosphorylated proteins, including the photolabeled proteins (Andersson and Porath, 1986). Once isolated, if sufficiently pure, the proteins can be digested and identified using matrix-assisted laser desorption ionization (MALDI) time-of-flight mass spectrometry (Karas and Hillenkamp, 1988).

### G-protein photolabeling

After incubation in serumless DMEM media for 1 h, five basal-turn explants were permeabilized in the dark for 5 min at RT with 25  $\mu$ M digitonin in serumless media containing 10  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]GTP- $\gamma$ AA with a specific activity of 10–20 Ci/mmol. In separate explants, cells were checked for viability 12, 24 and 48 h after incubation with digitonin, using the 0.4% Trypan Blue exclusion method, and no loss of viability was observed (data not shown). The supernatant was then removed and replaced for selected times with serumless media containing the same concentration of the isotope at room temperature. In test groups, the replacement media also contained 35  $\mu$ M gentamicin. Explants were incubated for 5 min or 1 h. The cultures were then exposed to hand-held UV light (254 nm) at a 2 cm distance for 2.5 min. The supernatant was then replaced with ice cold 2 mM dithiothreitol (DTT) in serumless media to terminate the reaction for 5 min. The DTT solution was then replaced with Laemmli buffer and 10%

SDS-PAGE was performed. After gel drying, autoradiography was performed (Jo et al., 1992). Photoactivity of the probe was confirmed by exposing the probe to serum GTP-binding protein and performing photolysis with a hand-held UV lamp. Negative controls were performed by treating explants for 48 h with 200  $\mu$ M gentamicin, thereby selectively killing HCs, 2 days prior to a second 5 min gentamicin treatment and G-protein labeling. Positive controls were performed by treating cardiac fibroblasts with 1  $\mu$ M bradykinin for 5 min, which activates an approximately 42 kD  $G_{\alpha}$  subunit.

### Immobilized $Al^{+3}$ -chelate affinity chromatography

Immobilized  $Al^{+3}$ -chelate affinity chromatography was performed as described by Shoemaker and Haley (1993). Iminodiacetic acid Sepharose 6B fast flow resin (1–2 ml; Sigma) was placed in a 5 ml syringe and washed with 10 ml of distilled water. Ten milliliters of 50 mM  $AlCl_3$  was then passed through the column and washed with 10–15 ml of distilled water. The column was then equilibrated with 100 mM ammonium acetate at pH 5.9. 40 basal turn explants previously photolabeled after treatment for 1 h with gentamicin were then homogenized in a small glass douncer containing 1 ml 100 mM ammonium acetate at pH 5.9. Negative controls contained explants treated only with serumless media. Homogenates were then passed through the charged resin column. The column was washed consecutively with 15 ml of 100 mM ammonium acetate at pH 5.9, 5 ml of 100 mM ammonium acetate containing 0.5 M NaCl at pH 5.9, and then 5 ml of 100 mM ammonium acetate at pH 5.9. Photolabeled proteins bound to the charged resin beads were then eluted with 5 ml of 100 mM ammonium acetate containing 10 mM  $KH_2PO_4$  at pH 8.0. The purified, photolabeled peptides eluted in the phosphate wash were pooled and then precipitated with 1:1 (500  $\mu$ l) isopropanol and tRNA (10  $\mu$ g/ml) at  $-20^{\circ}C$ . After overnight precipitation and pelleting, the pellets were solubilized using 1 $\times$  Tris-glycine sample buffer (Bio-Rad Laboratories, Chicago, IL, USA). Fifteen percent SDS-PAGE was then performed using two parallel wells for the experimental group and one well for the appropriate negative and positive controls. Pre-stained standards (Bio-Rad Laboratories) were used as well. The region containing the labeled protein was then cut from one experimental lane using a stainless steel scalpel, and the protein extracted for MALDI analysis.

### MALDI mass spectrometry

The gel piece was transferred to a 0.5 ml Eppendorf tube. Distilled water (0.4 ml) was added, the tube vortexed for 1 min, and the liquid discarded. Digestion buffer (25 mM ammonium bicarbonate, pH 8) was added, the mixture vortexed for 1 min, and the liquid discarded. Modified sequence grade trypsin (0.5  $\mu$ g; Promega) dissolved in digestion buffer (Promega, Madison, WI, USA) was then added to cover the gel piece at a trypsin concentration of 0.1  $\mu$ g/ $\mu$ l while minimizing evaporation of the digestion solution and  $Na^{+}$  and  $K^{+}$  salt precipitation. The gel piece was then incubated with agitation at  $30^{\circ}C$  overnight. The supernatant was then transferred to a new Eppendorf tube using a gel loading pipet tip. The gel piece was then re-extracted using 60% acetonitrile/0.01% TFA (enough to cover the piece) by agitating for 20 min at  $30^{\circ}C$ . The extract was then combined with the previous supernatant. This was repeated twice, while keeping the volume as low as possible. The pooled supernatants were then dried to approximately 10  $\mu$ l using a lyophilizer. The MALDI ion trap mass spectrometer is composed of an external MALDI ion source and a modified Finnigan ITMS electronics kit. Laser desorption/ionization was carried out at wavelength of 355 nm with 10 ns duration pulses. The matrix solution consisted of 2,5-dihydroxybenzoic acid (DHB) in 1:1 (v/v) ACN/ $H_2O$  (2 $\times$  dilution of a saturated solution of DHB in 1:1 (v/v) ACN/ $H_2O$ ). The mass spectroscopy samples were prepared by mixing 1  $\mu$ l of sample solution

with 1  $\mu$ l of matrix solution. The instrument was mass calibrated once a week.

### Procedure for protein identification

The peptide mixture produced by in-gel trypsin digestion of protein was analyzed directly by MALDI ion trap mass spectroscopy without further chromatographic separation or treatment. Trypsin peaks were used as internal standards to calibrate the mass spectrometer and enable high resolution spectroscopy with an error of 0.5 kDa mass units. After the mass spectroscopy peptide map was inspected, the mass units were entered and searched against predicted trypsin digest fragments of proteins in a mammalian protein database (Genbank, Oxford Molecular Group, Mountain View, CA, USA) using the program ProFound (Genomic Solutions, Lansing, MI, USA) with a tolerance of 0.2 kDa. Peaks that were not one third above background and those that matched the blank gel were not included. The search was carried out with constraints that include the mass range of the protein, the cleavage specificity of the digesting enzyme, and the originating species of the protein. A positive control sample containing 10  $\mu$ g/ml of human Ras (Sigma) and a blank sample were analyzed as well.

### Data analysis

Comparison of HC number after treatment with gentamicin and with different inhibitors was performed by using repeated measures ANOVA followed by LSD post hoc test (Stat View 5.0). Differences associated with  $P$ -values  $< 0.05$  were considered to be statistically significant. All data are presented as mean  $\pm$  S.E.

## RESULTS

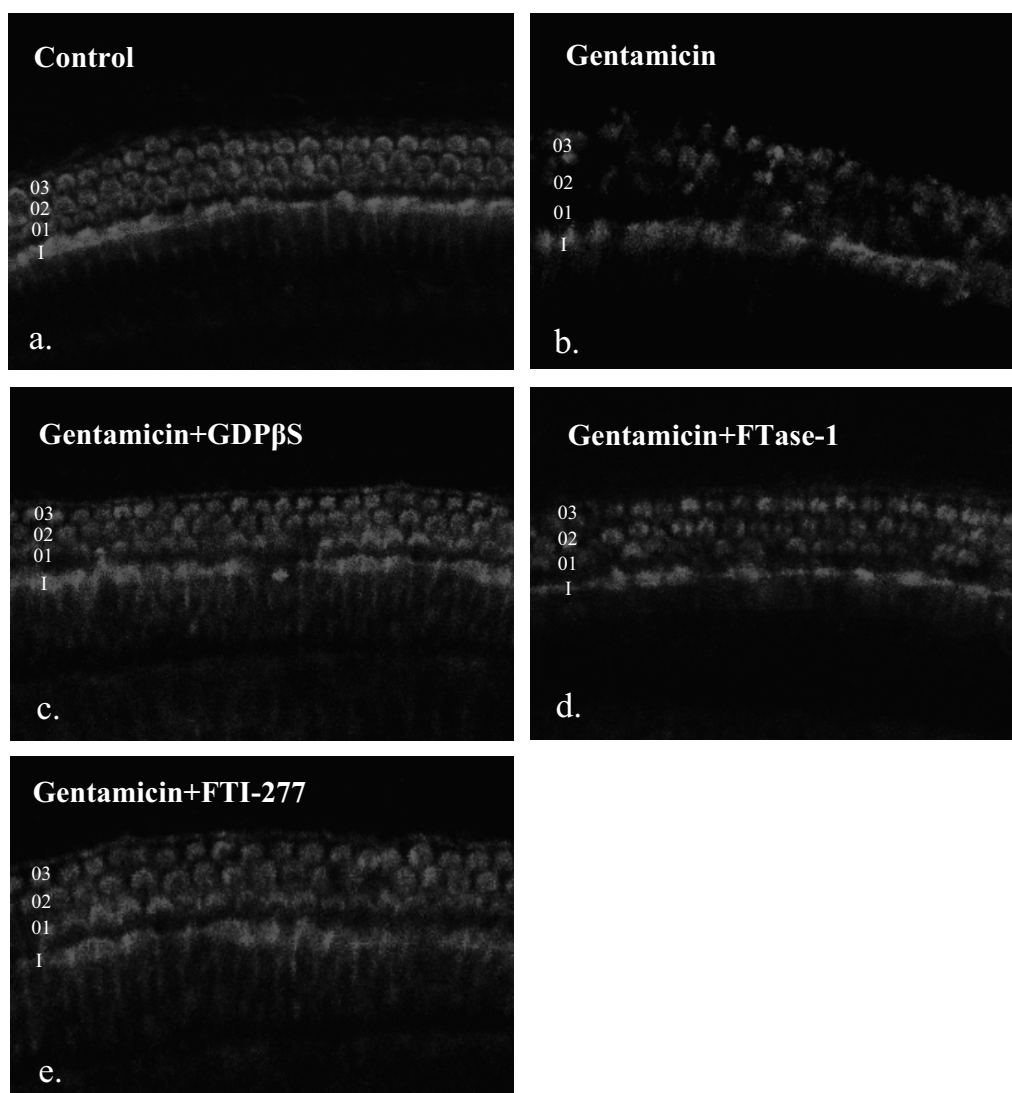
### *In vitro* ototoxicity

Phalloidin-rhodamine-stained control explants showed the normal configuration of a single row of IHCs and three rows of OHCs in the area of the basal turn after 48 h in culture (Fig. 1a). All of the HCs exhibited normal stereociliary morphology. Equivalent basal turn explants treated with 35  $\mu$ M gentamicin for 48 h showed significantly reduced HC survival (Fig. 1b). The first row of OHCs showed the greatest degree of damage, followed by the second row of OHCs and then the third row. Total loss of OHCs was about 50%. The IHCs were the most resistant to damage, showing only scattered loss.

### General G-protein inhibitor GDP- $\beta$ S protected auditory HCs

Treatment of basal turn explants with the general G-protein inhibitor GDP- $\beta$ S (1 mM) for 12 h prior to gentamicin exposure and during exposure for 48 h to 35  $\mu$ M gentamicin resulted in a significant increase ( $P < 0.01$ ) in the number of surviving OHCs as well as in the number of HCs that appeared morphologically normal compared with the group with gentamicin alone (Figs. 1c, 2a).

ADP- $\beta$ S did not protect the HCs against gentamicin toxicity ( $P = 0.216$ ; data not shown). Explants treated for 48 h with 1 mM GDP- $\beta$ S alone, also appeared normal (data not shown).



**Fig. 1.** a) Rhodamine–phalloidin staining of a cultured normal p5 rat OC basal turn explant. Note intact stereocilia of OHCs and IHCs. The OHCs (1–3) are in the uppermost three rows while the IHCs (I) are in the lowermost row. b) Representative p5 rat OC basal turn explant after 48h *in vitro* exposure to gentamicin. Note the extensive loss of OHCs with the IHCs being the most resistant to damage and the third row of OHCs being the second most resistant. c) Representative explant treated with GDP- $\beta$ S (1 mM) prior to and during exposure to gentamicin (35  $\mu$ M) *in vitro*. There was a significant ( $P < 0.01$ ) improvement in OHC survival compared with gentamicin alone. d) Explant treated with the Ftase-inhibitor B581 (50  $\mu$ M) prior to and during gentamicin exposure. Significant ( $P < 0.01$ ) protection of OHCs was observed. e) Explant treated with the Ftase-inhibitor FTI-277 (10  $\mu$ M) prior to and during gentamicin exposure *in vitro*. Again, significant ( $P < 0.01$ ) OHC protection was observed.

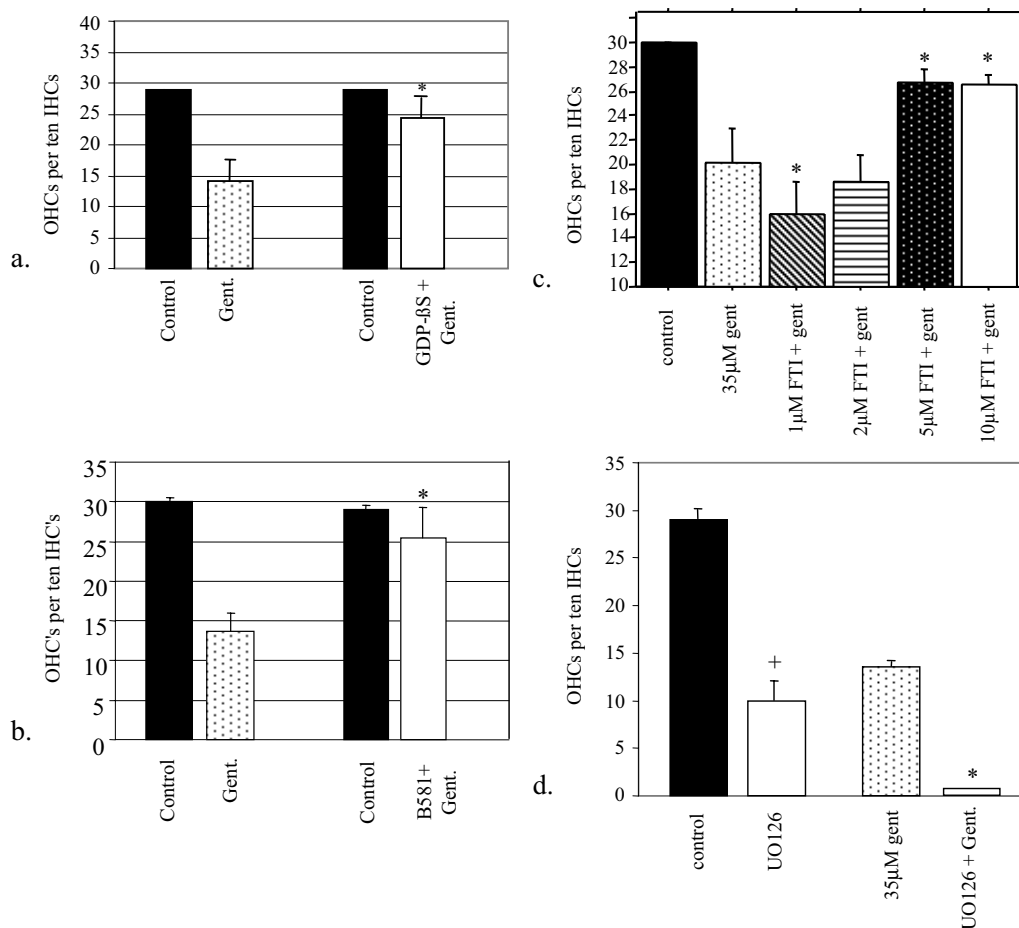
#### G-protein photoaffinity labeling in the OC after exposure to gentamicin

To determine the molecular weight of G-proteins that might be activated by gentamicin, basal turn explants were loaded with [ $\gamma$ - $^{32}$ P]GTP- $\gamma$ AA and then treated with gentamicin for 5 min or 1 h in the dark. After UV exposure, SDS-PAGE and autoradiography revealed that one or more approximately 21 kDa G-proteins were activated by gentamicin at both 5 min and 1 h (Fig. 3a). Negative controls revealed minimal photolabeling at 21 kDa in explants exposed to ototoxin-free media (Fig. 3a). Cardiac fibroblasts treated with 1  $\mu$ M bradykinin for 5 min showed a approximately 42 kDa labeled protein (data not shown). Pre-incubation of OC sam-

ples with 1 mM GDP- $\beta$ S prevented incorporation of the probe, while ADP- $\beta$ S did not. Explants pre-treated with 200  $\mu$ M gentamicin for 48 h in order to specifically destroy HCs prior to photolabeling experiments showed little or no incorporation of the azido-GTP probe during a subsequent gentamicin exposure, suggesting that the observed 21 kDa GTP binding is related to HCs (Fig. 3b).

#### Isolation and purification of 21 kDa band and MALDI analysis

Basal turn explants were loaded with [ $\gamma$ - $^{32}$ P]GTP- $\gamma$  AA as above and then treated with gentamicin for 1 h in the dark. After UV exposure, phosphorylated proteins were captured



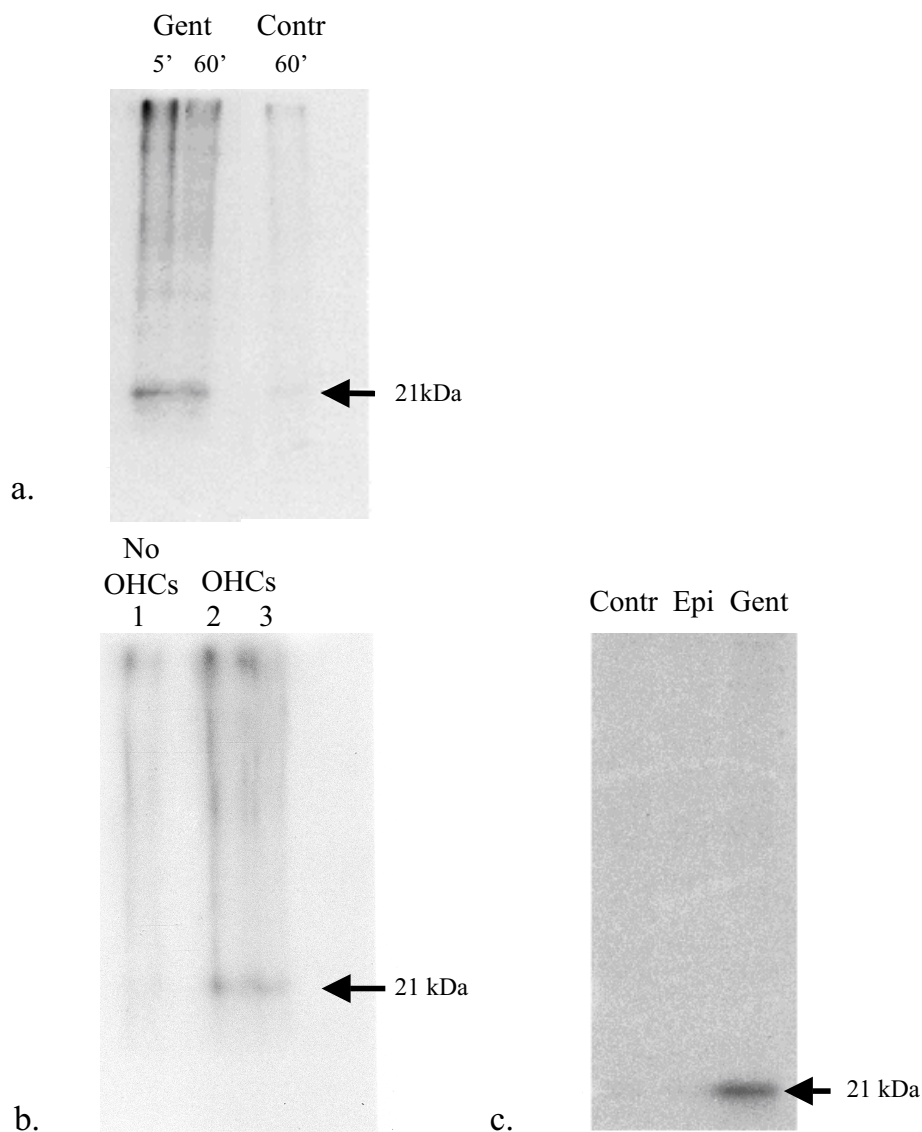
**Fig. 2.** a) Average OHC survival after exposure to gentamicin (35  $\mu$ M) *in vitro*, with or without 1 mM GDP- $\beta$ S ( $P < 0.01$ ). Control cultures received no gentamicin or inhibitor. The data are expressed as the mean number of surviving OHCs per 10 IHCs. The control columns represent separate control groups for each experimental condition. Vertical lines represent S.E.M. The error bars were too small to be seen in the control groups ( $29.7 \pm 0.3$  OHCs/10 IHCs). \* Indicates a significant difference from gentamicin alone. b) Average OHC survival after exposure to gentamicin, with or without B581 (50  $\mu$ M). The increase in survival induced by this inhibitor is significant ( $P < 0.01$ ). \* Indicates a significant difference from gentamicin alone. c) Average of OHC survival after exposure to gentamicin, with and without FTI-277 at the concentrations shown. HC survival is adversely affected at the lowest concentration (1  $\mu$ M;  $P < 0.05$ ) of FTI-277 compared with gentamicin alone, whereas at the highest concentrations (5, 10  $\mu$ M) FTI-277 protected HCs from gentamicin toxicity ( $P < 0.01$ ). \* Indicates a significant difference from gentamicin alone. d) Average basal turn OHC survival after exposure to gentamicin and/or the MEK inhibitor UO126 (1  $\mu$ M). There was a significant decrease in OHC survival after treatment with UO126 alone ( $P < 0.01$ ), and also when comparing gentamicin and UO126 versus gentamicin ( $P < 0.01$ ). + Indicates significant difference from control culture, \* indicates a significant difference from gentamicin alone.

by  $Al^{+3}$  IMAC and then eluted prior to resolution in two adjacent lanes by SDS-PAGE. Gel pieces were cut out in the 20–25 kDa range from one lane and trypsin digested prior to MALDI analysis. Autoradiography of the adjacent lane on the gel confirmed photolabeling of 21 kDa GTP binding protein (Fig. 3c).

High resolution MALDI analysis of the trypsin digested sample revealed a peptide map dominated by several peaks. When the peak spectrum was used to search the protein database, the optimal match was with c-H-Ras-p21, with 46% sequence coverage. The peptide peaks that matched c-H-p21-Ras produced a z score of 1.66 ( $P < 0.05$ ). MALDI of purified Ras positive control followed by database analysis showed the highest match to an H-Ras p21 protein mutant with Gln 61 replaced by Leu. The sequence coverage was 69% with a z score of 2.38 ( $P < 0.05$ ).

### The farnesyl-transferase inhibitors B581 and FTI-277 protect auditory HCs

To determine the functional role of Ras activation in gentamicin-mediated OHC death, basal turn explants were pre-treated with 50  $\mu$ M B581 or 10  $\mu$ M FTI-277 for 12 h prior to exposure to gentamicin (in addition to the inhibitor). We observed a significant ( $P < 0.05$ ) increase in OHC survival in explants treated with either inhibitor, compared with explants treated with gentamicin alone (Figs. 1d, e, 2b). In the case of FTI-277 we observed a dose-dependent effect in OHC survival (Fig. 2c). The lowest dose of FTI-277 (1  $\mu$ M) was associated with significantly more OHC loss than observed with gentamicin alone ( $P < 0.01$ ; Fig. 2c). However, at the highest dosages (5 and 10  $\mu$ M) significant OHC protection occurred ( $P < 0.01$ ; Fig. 2c). Explants



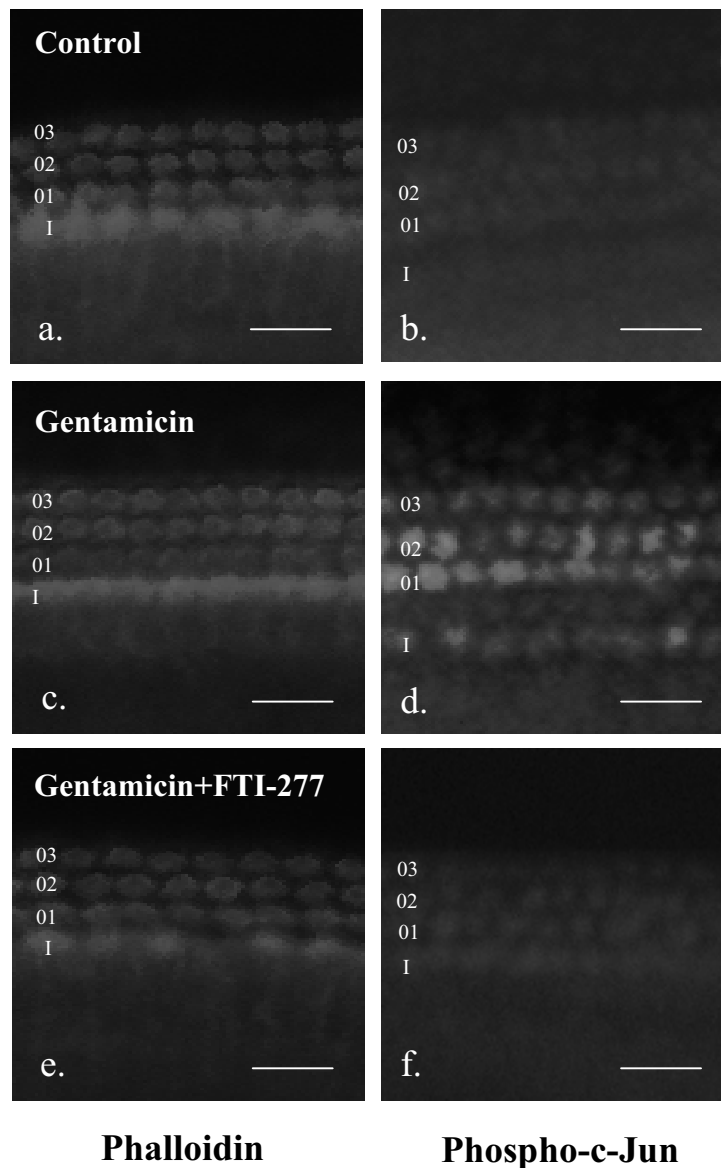
**Fig. 3.** a) Autoradiography of proteins from control P5 rat OC basal turn explants and explants treated with 35  $\mu$ M gentamicin for 5 min or 1 h. Explants were exposed to UV light to induce photochemical cross-linking of [ $\gamma$ - $^{32}$ P] GTP $\gamma$  AA to activated G-proteins prior to SDS-PAGE, which revealed a 21 kDa protein. Negative control explants maintained only in culture media for 1 h revealed minimal photolabeling at 21 kDa. b) Autoradiography of P5 rat OC explants exposed to gentamicin. Explants pre-treated with 200  $\mu$ M gentamicin for 48 h in order to specifically kill HCs and then allowed to recover for 48 h prior to a 5 min treatment with gentamicin and photolabeling showed minimal incorporation of the azido-GTP probe (lane 1). Normal explants (lanes 2, 3) showed GTP binding of 21 kDa protein in response to gentamicin. c) Autoradiography of SDS-PAGE after  $Al^{+3}$  IMAC purification of proteins from OC explants. There is no protein labeling in the negative control explants or in explants treated with 1  $\mu$ M epinephrine. Proteins from explants treated with gentamicin showed strong labeling at 21 kDa. An identical lane was cut out and protein extracted for MALDI analysis.

treated with 10  $\mu$ M FTI-277 alone were normal (data not shown).

#### FTI-277 attenuates JNK signaling in ototoxicity

To determine whether the protection of OHCs by Ras inhibition using FTI-277 was associated with an attenuation of the JNK signaling pathway, explants were either exposed to gentamicin or pretreated with 10  $\mu$ M FTI-277 and then exposed to gentamicin plus FTI-277 for 20 h prior to phospho-c-Jun immuno-labeling. Control explants, exposed to culture medium alone, showed no

detectable immunostaining of HC nuclei (Fig. 4b). Basal turn explants of the OC exposed to 35  $\mu$ M gentamicin for 20 h and immunostained showed strong phospho-c-Jun immunolabeling of OHC nuclei, and a lesser labeling of IHC nuclei (Fig. 4d). There was no labeling of supporting cell nuclei. Explants treated with FTI-277 prior to and during gentamicin treatment showed little or no immunostaining (Fig. 4f). Because of the reduced time of exposure to gentamicin, all explants when stained with phalloidin-rhodamine showed one row of IHCs and three rows of OHCs (Fig. 4a, c, e).



**Fig. 4.** a, b) Basal turn p5 OC explants maintained in culture medium alone (Control) for 32 h show the normal configuration of the OC in phalloidin labeled images (a), and no phospho-c-Jun immunolabeling (b). O1, O2, O3: row 1, 2, and 3 of OHCs, I: IHCs. Bar=20  $\mu$ m. c, d) Basal turn explant maintained in culture for 12 h and then exposed to 35  $\mu$ M gentamicin for 20 h shows normal morphology in the phalloidin-labeled image (c), and strong staining of the OHC nuclei and weaker labeling of IHC nuclei by the phospho-c-Jun antibody (d). Bar=20  $\mu$ m. e, f) Basal turn explant pretreated with FTI-277 (10  $\mu$ M) for 12 h and FTI-277 plus gentamicin (35  $\mu$ M) for 20 h shows normal OC in the phalloidin-rhodamine image (e), and little or no phospho-c-Jun immunolabeling (f). Bar=20  $\mu$ m.

#### Auditory HCs and MEK: studies with U0126

To determine whether Ras signaling via MEK and the ERK MAPK played a role in OHC toxicity, basal turn explants were exposed to gentamicin with or without a MEK inhibitor. Inhibitor control explants were exposed to the MEK inhibitor alone. Treatment of basal turn explants with the MEK inhibitor U0126 alone resulted in significant ( $P<0.05$ ) OHC loss (Fig. 2d). When gentamicin was added, both OHCs and IHCs were completely lost ( $P<0.001$ ; Fig. 2d). The control compound U0124 had no effect on normal or gentamicin-treated OC (data not shown).

#### DISCUSSION

We have previously shown that the growth factor FGF-2, whose receptor often activates G-proteins, protects OHCs against ototoxins (Low et al., 1996). As a preparatory step in assessing the role of G-proteins in this protection, we treated basal turn OC explants with a general G-protein inhibitor prior to and during gentamicin exposure. Against our expectations, explants treated with GDP- $\beta$ S revealed significantly improved HC survival, even in the absence of FGF-2, suggesting that inhibition of G protein activity itself can protect HCs from this toxin.



Intracellular GDP- $\beta$ S presumably inactivates G-proteins which would otherwise initiate cell death after exposure to gentamicin. The fact that ADP- $\beta$ S, a structural analog of GDP- $\beta$ S with similar charge, did not significantly improve HC survival after gentamicin exposure supports the interpretation that GDP- $\beta$ S acts through intracellular inhibition of G-protein activity as opposed to extracellular neutralization of the toxin or a nonspecific intracellular activity of nucleotides. [ $\gamma$ - $^{32}$ P]GTP- $\gamma$ AA photolabeling studies indicated that, relative to untreated explants, gentamicin-treated explants exhibit increased GTP binding activity by proteins in the 21 kDa range, such as members of the Ras or Rho families.

Exposure to stressful stimuli induces small G-protein activation in other cell systems. Interestingly, this activation can be either protective or damaging depending upon, for one thing, the length of stress application. For example, 5–10 min of exposure to free radicals in Jurkat T-cells has been shown to induce Ras activation directly by redox modification of amino acid residue Cys<sup>118</sup> (Lander et al., 1997) which in turn results in Ras binding to the effector PI3K (Deora et al., 1998). The serine-threonine kinase Akt/PKB can subsequently be activated leading to increased BAD phosphorylation, resulting in initial protection of the cell (Basu et al., 1998; Joneson and Bar-Sagi, 1999). Longer term exposure to stress has been shown to initiate cellular damage via small G-protein activation and the JNK pathway (Kharbanda et al., 2000). For example, Cos-7 cells transfected with dominant negative Ras are protected from apoptosis induced by long-term exposure to ceramide (Basu et al., 1998).

The results of MALDI and peptide matching indicated that H-Ras is activated in the OC after brief toxin exposure. Other isoforms of Ras in addition to members of the Rho family do not appear to be activated at this early time, according to the MALDI analysis. The potential role of this Ras activation in HC toxicity was explored with Ftsase inhibitors.

Ftsase inhibitors block the activity of Ras by inhibiting the prenylation that is required for membrane insertion of Ras, which is in turn necessary for downstream Ras signaling (see Leonard, 1997 for a review). Within the Ras superfamily, Ftsase inhibition is specific to Ras proteins, since Rho isoforms (with the exception of RhoB) and Rac are geranylgeranylated rather than farnesylated. FTI-277 does not affect geranylgeranylation at concentrations up to at least 10  $\mu$ M, the highest dose used in the present study, suggesting that the effects of Ftsase inhibitors within the Ras superfamily are limited to Ras isoforms. However, it should be noted that in addition to Ras, other proteins such as lamin B,  $\gamma$ -transducin, RhoB, and CENPs are also farnesylated. It is therefore possible that inhibition of these proteins is responsible for the protection of HCs from gentamicin. The lack of prior data linking them to HCs or to ototoxicity makes this less likely.

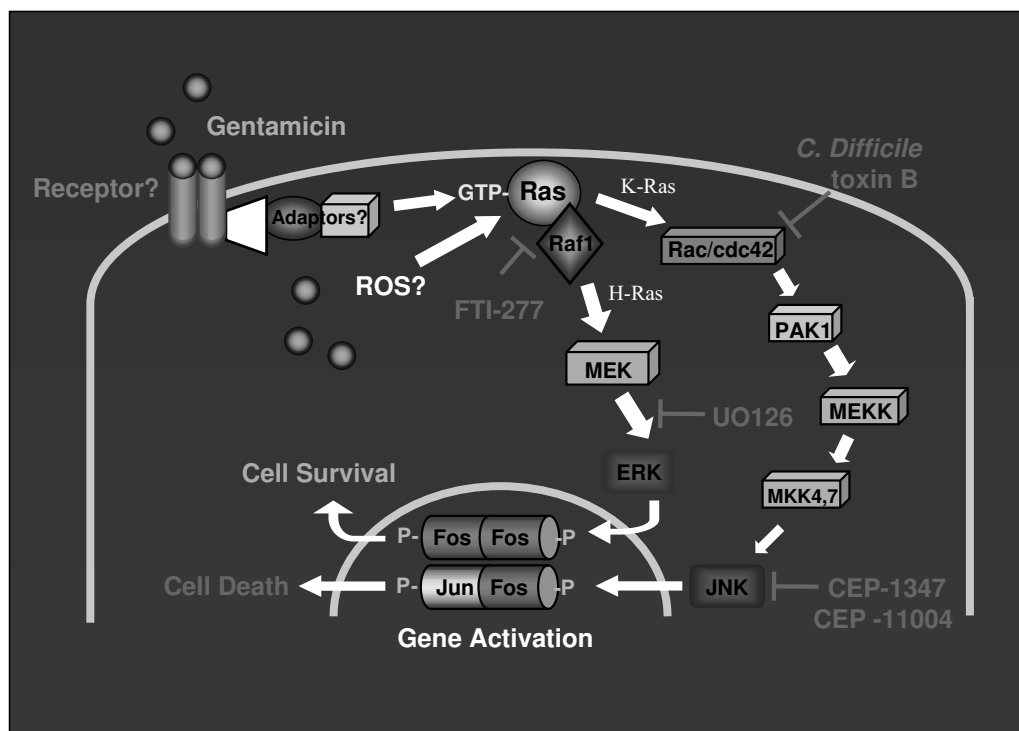
Interestingly, a dose response study of HCs to FTI-277 showed that HC survival is adversely affected at low concentrations (1  $\mu$ M) of this reagent, but that protection occurs at higher dosages (5–10  $\mu$ M). This suggests that Ras

pathways contributing to HC survival are selectively inhibited at lower concentrations while Ras-dependent pathways contributing to HC death are selectively inhibited at higher concentrations (10  $\mu$ M). Potentially relevant to this issue, FTI-277 has been shown to have a dose-dependent specificity for the different Ras isoforms, H-Ras, N-Ras and K-Ras. H-Ras processing is inhibited at concentrations as low as 10 nM of FTI-277 and is almost completely blocked at 3  $\mu$ M (Casey et al., 1989; Lerner et al., 1995). Inhibition of N-Ras processing requires 5  $\mu$ M of FTI-277 while complete inhibition of K-Ras requires 10  $\mu$ M (Casey et al., 1989; Lerner et al., 1995). Thus, the data from our dose-response study suggest that low concentrations of FTI-277 may enhance OHC death through inhibition of H-Ras, while higher concentrations of FTI-277 may protect OHCs through inhibition of K-Ras and/or N-Ras.

This concept is supported by mounting data in the literature which indicate that the different Ras isoforms generate specific functional endpoints (Wolfman, 2001). Disruption of the gene encoding K-Ras results in a lethal embryonic phenotype while H-Ras and N-Ras knockouts are viable, indicating significant differences between the isoforms in regulating development (Umanoff et al., 1995; Johnson et al., 1997; Koera et al., 1997; Esteban et al., 2001). H-Ras is frequently involved in MEK/ERK activation via Raf 1. K-Ras appears to be more involved in Rac activation than the other isoforms. Overexpression of K-Ras increases Rac/cdc42 accumulation, which in turn leads to more membrane ruffling and faster cell migration when compared with cells transfected with G12V-H-Ras (Walsh and Bar-Sagi, 2001). N-Ras appears to provide steady-state survival signals through PI3K, Akt and mitochondrial Bcl-2 family proteins (Wolfman and Wolfman, 2000).

We found that MEK inhibitors also influenced HC survival, proving to be toxic to OHCs even without gentamicin. When they were used in combination with gentamicin, strong potentiation of toxicity was observed, and all OHCs and IHCs were lost. This suggests that normal OHCs and IHCs depend upon MEK activation for survival, and that MEK activity opposes cell death during toxin treatment. Ras is often an upstream activator of MEK, suggesting the Ras/Raf/MEK/ERK MAPK pathway. However, the observation that the MEK inhibitor alone but not FTI-277 alone is toxic to OHCs indicates that there may be other upstream activators of MEK such as protein kinase C, Rap1 or RafB (via crkL and C3G) that act independently of Ras (McCubrey et al., 2000) in normal HCs. In damaged HCs, H-Ras may contribute to MEK activation as it does in other cell types (Yamamoto et al., 1999).

With respect to HC damage, we recently observed inhibition of gentamicin-induced HC damage with *Clostridium difficile* toxin B, a potent inhibitor of Rho GTPases including Rac/cdc42 (Bodmer et al., 2002a). Rac/cdc42 could provide a link between K-Ras and JNK signaling. Other possible downstream targets of Ras exist, including p38 and PI<sub>3</sub>K. However we have found that relatively high concentrations of inhibitors of these pathways did not re-



**Fig. 5.** A simplified model of signal transduction in gentamicin-induced HC damage, based on the results of this and other studies. The mode of initial interaction between the HC and gentamicin is not known. It may include a receptor, possibly with adaptor molecules leading to Ras activation, and/or entry of gentamicin into the cell followed by generation of reactive oxygen species (ROS) that activate Ras. Immediate activation of H-Ras, blocked by low dosages of FTI-277, leads to MEK/ERK mediation of cell survival, which in turn can be blocked by UO126. Prolonged activation of K-Ras, blocked by high dosages of FTI-277, leads to activation of Rac/cdc42, which can be inhibited by *C. difficile* Toxin B. Through intermediaries this activates JNK, which can be blocked by CEP-1423 or CEP 11003, leading to cJun phosphorylation and cell death.

duce gentamicin ototoxicity in our model system (data not shown).

A model for Ras activity in the gentamicin-exposed OHC can be inferred from our data and from the results of others (Fig. 5). In this model, initial ototoxin exposure activates H-Ras, which then promotes HC survival through increased activation of MEK and the ERK MAPK. Thus, inhibition of H-Ras with low concentrations of FTI-277, or inhibition of MEK with UO126 enhances OHC death. Prolonged ototoxin exposure activates K-Ras, which promotes HC death via increased activity of JNK. Thus, inhibition of K-Ras with high concentrations of FTI-277, inhibition of Rac/cdc42 (Bodmer et al., 2002b) or inhibition of JNK (Pirvola et al., 2000; Bodmer et al., 2002b) can protect HCs from aminoglycoside exposure. The observation that high-dose FTI-277 decreases c-Jun phosphorylation in HCs due to gentamicin exposure is consistent with this interpretation.

As noted above, Ras has been shown to have variable functions depending on the environmental and genetic conditions of a cell. Ras is not alone in having variable functions. Dual functions for the regulation of cell survival, proliferation, differentiation or apoptosis have been shown for c-Myc (Evan and Littlewood, 1998), and c-Fos (Colotta et al., 1992). The evidence in this study points toward Ras playing roles both in HC survival and cell death.

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